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*in Arabidopsis. In Methods in Arabidopsis*  
21.

J.M., GOODMAN H.M., KOORNNEEF  
MEYEROWITZ B.M., 1993. An integrated  
J., 3, 745-754.

CAROCHE M., MOISAN A., JOURJON  
JER D., GIRAUDAT J., GUIGLEY F.,  
COXS R., GRELLET F., DELSENY M.,  
BLECK J., PHILIPPS G., AXELLOS M.,  
An inventory of 1152 expressed sequence  
in *thaliana*. *Plant J.*, 4 (6), 1051-1061.

, SCHMIDT R., CNOPS G., DHAN C.,  
LANEY L., SOMERVILLE C., 1991.  
Third of the *Arabidopsis* genome. *Plant J.*

Mapping RFLP and phenotypic markers in

DOS W.D.B., HANGE B.M., GOODMAN  
A map of *Arabidopsis thaliana*. *Plant Cell*,

2, 9, 111-127.

Construction of an overlapping YAC library of  
341-351.

## Marker-assisted backcrossing: a practical example

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### Summary

That molecular markers allow fast recovery of recurrent parent genotype in backcross programs is undisputed. Restriction Fragment Length Polymorphisms (RFLP's) were used in maize to introgress by backcross a transgene construct, containing phosphinothricin resistance and insecticidal protein genes, from a transformed parent into an elite inbred line. At each generation plants carrying the transgene construct were selected based on their phosphinothricin resistance, and further characterized with RFLP's. Both maximum recovery of recurrent parent genotype and minimum linkage drag were taken into account for marker-based selection. Embryo rescue was used to shorten generation time. Progress towards recurrent parent genotype was spectacular. Levels of recurrent parent genotype recovery which would normally be observed, in the absence of selection, in the BC<sub>5</sub> generation were obtained at the BC<sub>3</sub> generation, about one year after BC<sub>1</sub> seeds had been planted. Besides the evidence already provided by RFLP's, phenotypic evaluation of the backcross-derived near-isogenic lines will constitute an additional check of the completeness of the conversion.

### Introduction

Backcrossing has been a common breeding practice for as long as elite germplasm has been available. It has mainly been used to introgress single Mendelian traits, such as disease resistances or quality factors, into elite germplasm (Allard 1960; Hallauer and Miranda 1981). One of the most attractive attributes of backcrossing is that it allows to perform targeted modifications without disrupting the existing overall genetic balance of the recurrent parent.

However, production of fully converted near isogenic lines through classical backcrossing procedures is a lengthy procedure. If at all possible. Theoretically, a minimum

### APPENDIX 1

of seven classical backcross generations are required to recover more than 99% of recurrent parent genotype, assuming no linkage drag. The attractiveness of classical backcross procedures is therefore substantially diminished for crops, such as maize (*Zea mays* L.), where the turn-over of elite cultivars is very fast. In addition, full recovery of recurrent parent genotype is usually not achieved through classical backcrossing, which may result in deleterious agronomic effects. Murray *et al.* (1988) reported about 90% recurrent parent genotype recovery in two BC<sub>10</sub>-equivalent conversions (A632Ht and A632Rp) of the maize line A632. The conversions had retained respectively 4 and 7 donor fragments in addition to the one carrying the gene of interest.

Reduction in the number of backcross generations needed to obtain fully converted individuals has been shown theoretically, or from simulations, to be achievable through the use of molecular markers (Tankaley *et al.* 1989; Hospital *et al.* 1992; Jarboe *et al.* 1994). Because they provide thorough characterization of the genetic variability at each backcross generation, markers allow to take full advantage of this variability by applying the highest possible selection intensity.

Efficiency of marker-assisted backcrossing was investigated through an experiment aimed at introgressing a single genetic factor (a transgene construct) from a donor into a recipient maize line.

## Materials and methods

### Plant Material

A hemizygous transgenic maize line of Lancaster origin was used as donor parent to introgress its transgene construct, through repeated backcrossing, into a recipient parent from the Stiff Stalk germplasm group. Both parents are proprietary elite lines. The transgene construct carries both a phosphinothricin resistance gene and synthetic genes encoding the entomotoxic fragment of the CryIA(b) *Bacillus thuringiensis* protein (Kozic *et al.* 1993). Transformation was achieved through microprojectile bombardment (Kozic *et al.* 1993) and resulted in a single insertion (*Bt* locus), on chromosome 1 (Figure 1).

### Backcross protocol

The F1 progeny of the cross between the donor and the recipient was screened for the presence of the transgene construct by applying Basta, a phosphinothricin-based herbicide, onto each plant. Resistant individuals were then used to generate BC<sub>1</sub> progeny.

For each backcross generation, except the BC<sub>4</sub>, individuals were planted in multipots and sprayed with Basta to eliminate those which did not carry the transgene construct. To avoid the stress resulting from treatment with Basta, BC<sub>4</sub> plants carrying the transgene construct were identified using Southern blots probed with the *pat* and *Bt* genes. Resistant plants were transplanted in an open-soil greenhouse and leaf-sampled for molecular marker

analyses. Results of marker analysis were used to select plants for flowering. A single plant was rescued and transferred onto the embryos first underwent a growth culture medium, before being average, four months.

### Molecular marker analysis

Restriction Fragment Length polymorphism (RFLP) genotypes in all four genetic backgrounds were determined using chemiluminescent techniques. Loci were chosen from among those that provided coverage of the entire genome, contained two loci tightly linked recombination units away (Figure 1), BC<sub>4</sub>+1 generation comprised by or tightly linked ones, and added selected BC<sub>4</sub> plant was heterozygous independent reference population generation.

### Selection procedure

At each generation plants of recurrent-parent-genotype and attempt to integrate both criteria. Missing values were not included contributed to the selection process. Best ranking one of those for which the BC<sub>4</sub> selection was available.

## Results and discussion

### Selection for the gene of interest

The observed segregation was significantly different ( $P < 0.05$ ).

### Recurrent parent genotype

Statistics for the genotype were performed taking the whole genome backcross-derived plant thereof.

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ops, such as maize (*Zea mays* L.).  
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(A632Ht and A632Rp) of the maize  
and 7 donor fragments in addition to

needed to obtain fully converted  
ations, to be achievable through the  
al *et al.* 1992; Jarboc *et al.* 1994).  
genetic variability at each backcross  
variability by applying the highest

investigated through an experiment  
the construct) from a donor into a

origin was used as donor parent to  
backcrossing, into a recipient parent  
are proprietary elite lines. The  
distance gene and synthetic genes  
*luc* thuringiensis protein (Kozziel *et*  
projectile bombardment (Kozziel *et*  
chromosome 1 (Figure 1).

the recipient was screened for the  
phosphinothricin-based herbicide.  
generous BC<sub>1</sub> progeny.

Individuals were planted in multipots  
carry the transgene construct. To  
BC<sub>1</sub> plants carrying the transgene  
the *pat* and *Bt* genes. Resistant  
leaf-sampled for molecular marker

analyses. Results of marker analyses were made available at the latest two weeks after  
flowering. A single plant was selected, of which all backcross-derived embryos were  
rescued and transferred onto tissue culture medium. Plants that developed from these  
embryos first underwent a greenhouse acclimation phase, while still growing on tissue  
culture medium, before being transplanted into multipots. Backcross cycles lasted, on  
average, four months.

#### Molecular marker analyses

Restriction Fragment Length Polymorphisms (RFLP's) were used to establish  
genotypes in all four generations. RFLP detection involved either radioactive or  
chemiluminescent techniques. For the BC<sub>1</sub> generation, 61 marker-enzyme combinations  
were chosen from among those revealing polymorphism between donor and recipient. They  
provided coverage of the entire genome, defining intervals of about 25 cM in size, and  
contained two loci tightly linked to the *Bt* locus, CG320 and CG415, respectively 5 and 16  
recombination units away (Figure 1). For subsequent generations, markers analyzed in the  
BC<sub>n+1</sub> generation comprised both those for which the selected BC<sub>n</sub> plant was heterozygous,  
or tightly linked ones, and additional ones located in chromosomal segments for which the  
selected BC<sub>n</sub> plant was heterozygous (Table 1). Marker map positions were obtained from  
independent reference populations and confirmed by analysis of segregation in the BC<sub>1</sub>  
generation.

#### Selection procedure

At each generation plants were ranked based both on the percentage of homozygous  
recurrent-parent-genotype and on the extent of linkage drag around the *Bt* locus, in an  
attempt to integrate both criteria. Plants for which two or more adjacent markers had  
missing values were not included in the analyses. Success or failure of the pollinations also  
contributed to the selection procedure. One single plant was selected at each generation: the  
best ranking one of those for which a backcross progeny of size 100 or more (50 or more  
for the BC<sub>3</sub> selection) was available.

### Results and discussion

#### Selection for the gene of interest

The observed segregation ratios for phosphinothricin resistance (Table 1) were not  
significantly different ( $P < 0.05$ ) from the expected 1:1, as shown by Chi-square tests.

#### Recurrent parent genotype recovery

Statistics for the genotyped plants are summarized in Table 1. Calculations were  
performed taking the whole genome into account, including the *Bt* locus. The "perfect"  
backcross-derived plant therefore counts one heterozygous chromosome segment, that

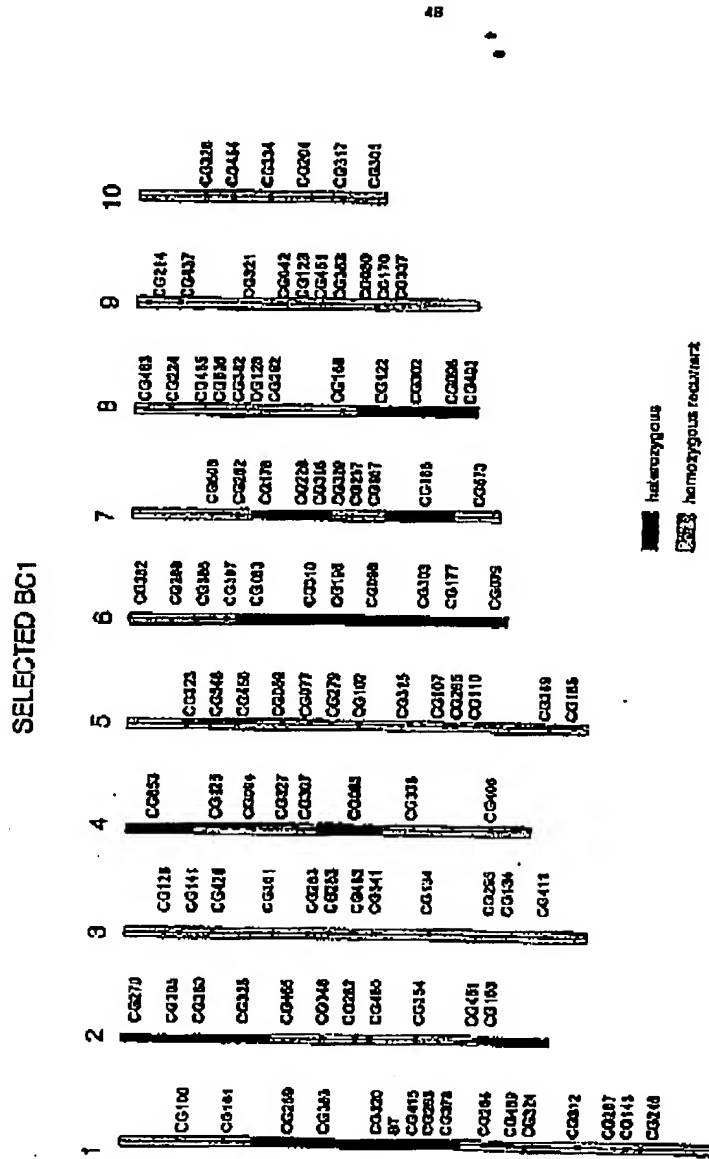
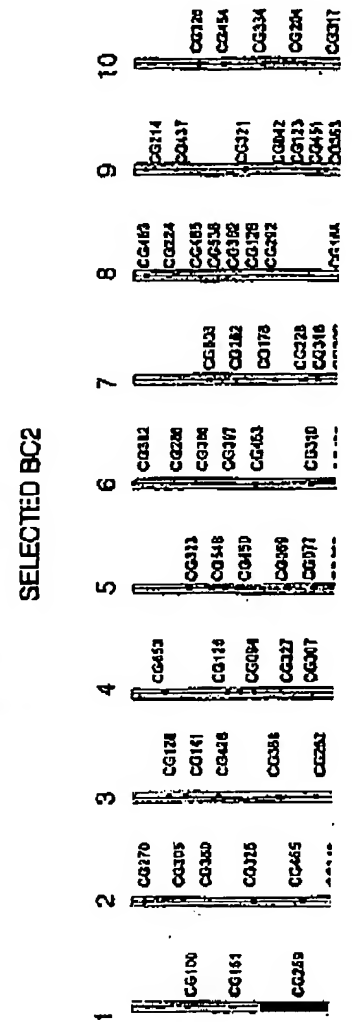


Figure 1-41: Genetic maps of the backcross-derived individuals selected in the first four generations of a marker-assisted backcross program. The locus to be introgressed (*Bt*) is located on chromosome 1.



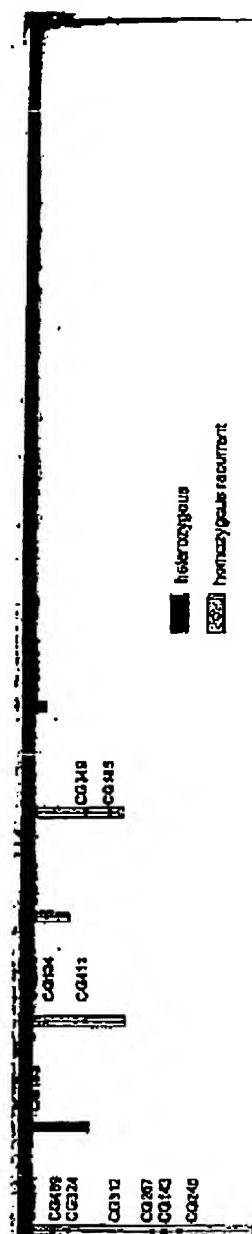


Figure 1-a: Genetic maps of the backcross-derived individuals selected in the first four generations of a marker-assisted backcross program. The locus to be introgressed (Bt) is located on chromosome 1.

### SELECTED BC2

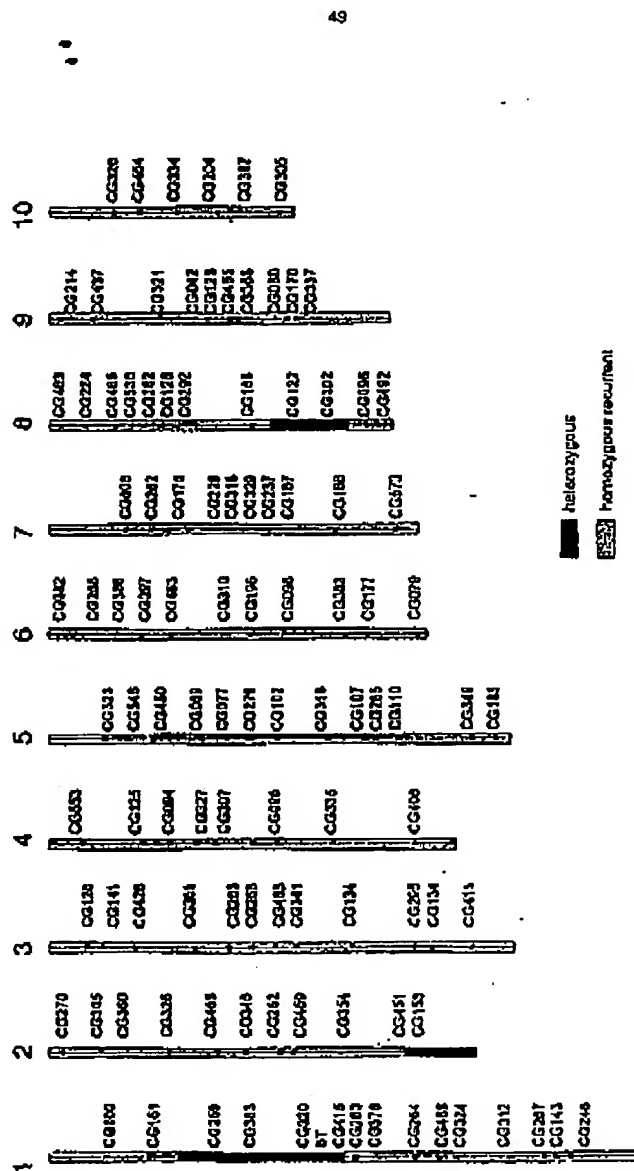


Figure 1-b: Genetic maps of the backcross-derived individuals selected in the first four generations of a marker-assisted backcross program. The locus to be introgressed (Bt) is located on chromosome 1.

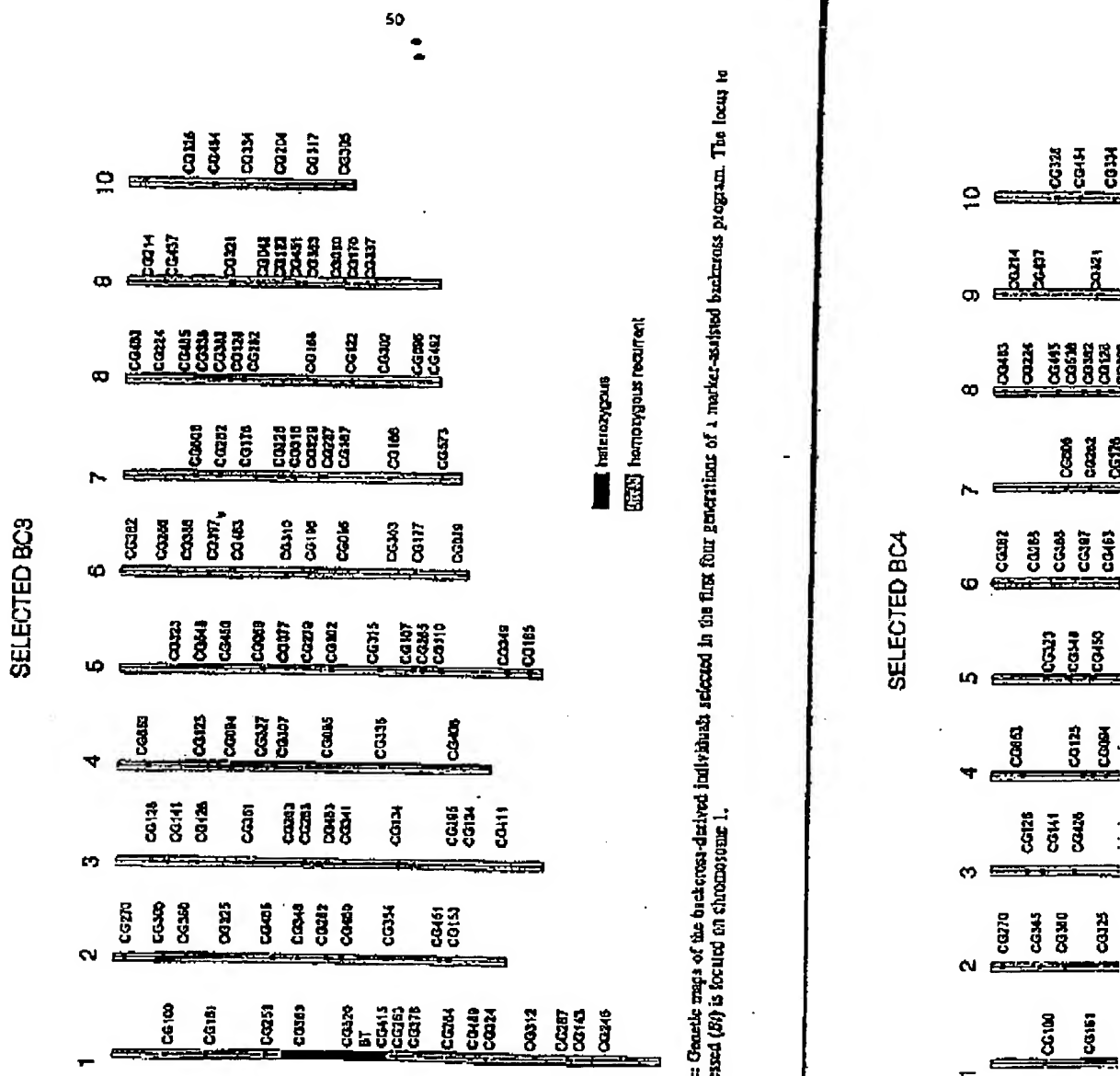


Figure 1— Genetic maps of the backcross-derived individuals selected in the first four generations of a marker-assisted backcross program. The locus to be introgressed (*Bt*) is located on chromosome 1.

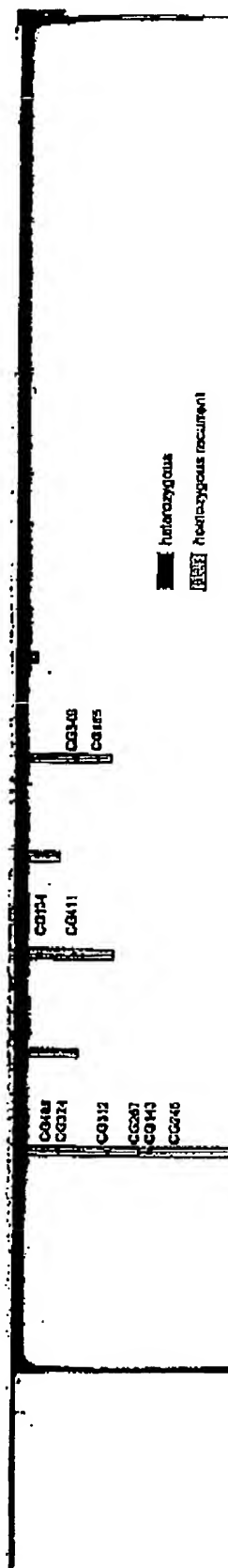


Figure 1-c: Genetic maps of the backcross-derived individuals selected in the first four generations of a marker-assisted backcross program. The locus to be introgressed (*Bt*) is located on chromosome 1.

# SELECTED BC4

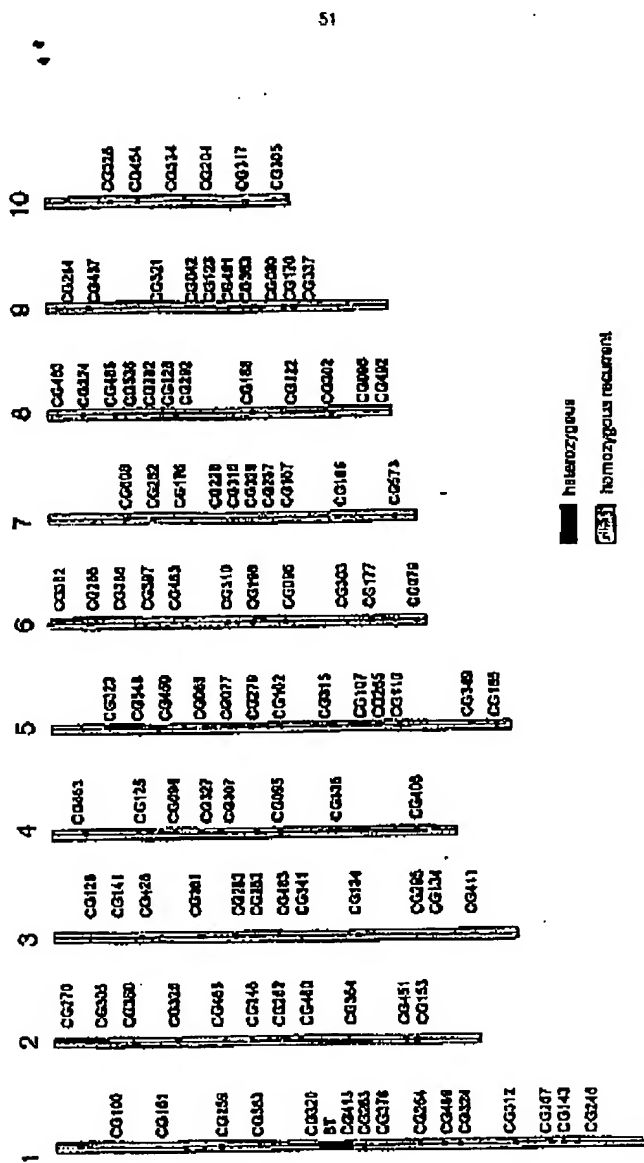


Figure 1-d: Genetic maps of the backcross-derived individuals selected in the first four generations of a marker-assisted backcross program. The locus to be introgressed (*Bt*) is located on chromosome 1.

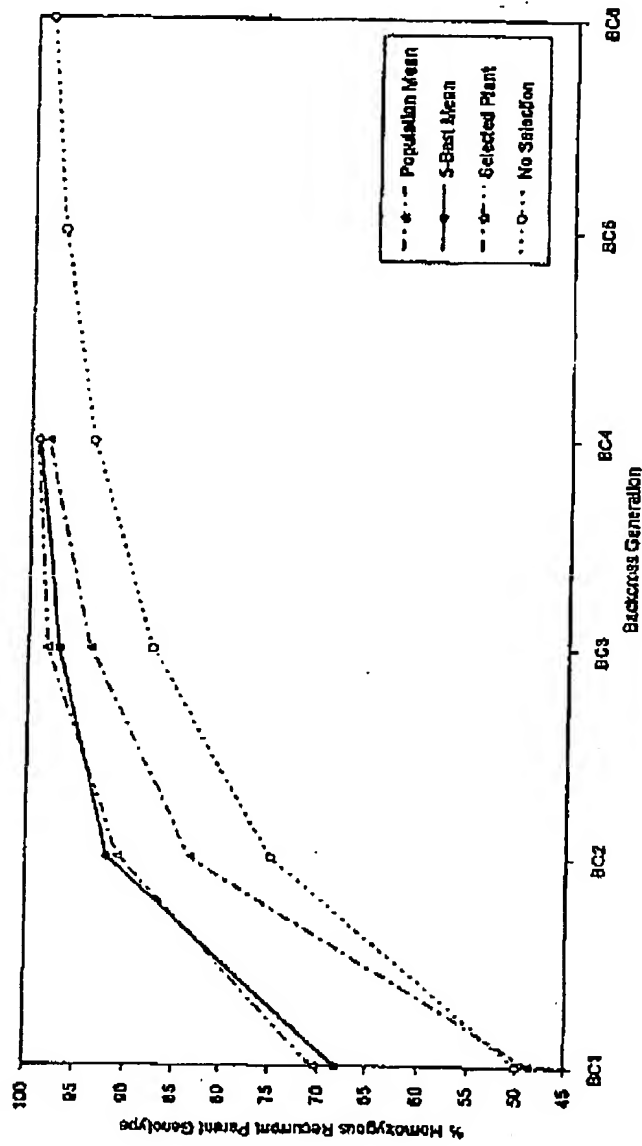


Figure 2: Recovery of recurrent parent genotype through backcrossing, with or without marker-assisted selection

Table 1: Proportion and characteristics of plants carrying the genes of interest, in the first four generations of a marker-assisted backcross program.

Generation	% rhizoglucosylase		RFLP genotyping		no plants		% homozygous recurrent		no heterozygous	
	BC1	BC2	BC3	BC4	BC5	BC6	BC1	BC2	BC3	BC4



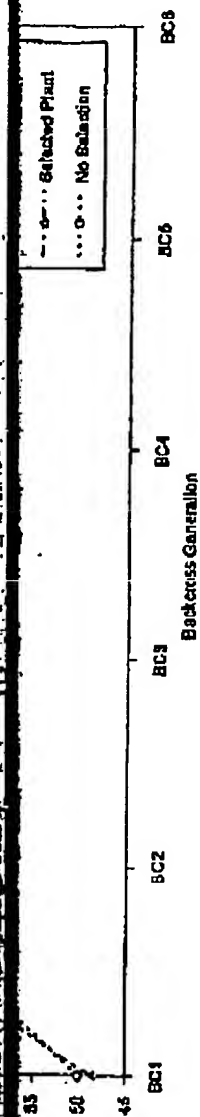


Figure 2: Recovery of recurrent parent genotype through backcrossing, with or without marker-assisted selection

Table 1: Proportion and characteristics of plants carrying the genes of interest, in the first four generations of a marker-assisted backcross program.

generation	% phosphinotriazin resistant plants	RFLP genotyping			nb plants analyzed *	% homozygous recurrent parent genotype			nb heterozygous chimeric segments ***		
		nb plants	nb RFLP	nb alleles		mean	std dev	selected plant	mean	std dev	selected plant
BC1	48.05	98	61	5860	87	48.72	10.35	68.31	11.01	2.17	7.76
BC2	44.55	61	22	1342	50	63.42	5.84	61.98	5.03	1.84	3.20
BC3	48.32	72	10	720	71	63.33	1.85	60.62	2.30	0.71	1.80
BC4	-	26	3	76	26	68.23	0.49	69.05	1.00	0.00	1.00

\* Plants for which two or more adjacent markers had missing values were not included in the analyses.  
 \*\* Mean value of the five individuals having the five highest percentages of homozygous recurrent parent genotype.  
 \*\*\* Including the segment carrying the transgene construct.

comprising the *Bt* locus. It also displays 99.36% of homozygous recurrent-parent-genotype. The remaining 0.64% corresponds to the average relative length of the chromosome segment containing the *Bt* locus, which depends on the two flanking markers chosen.

The mean percentage of homozygous recurrent-parent-genotype of the  $BC_1$  generation was slightly lower than the expected 50%. This can be explained by linkage drag around the *Bt* locus, given that this percentage was computed based only on plants selected for heterozygosity at the *Bt* locus. For all other backcross generations the mean percentage of homozygous recurrent-parent-genotype was much higher than what would have been observed, should no selection have been done (Figure 2).

The percentage of homozygous recurrent-parent-genotype of the selected plant (Table 1) and the average of the five largest values (Table 1) were always very similar to one another, and much superior to the population mean value (Figure 2). The percentage of homozygous recurrent-parent-genotype of the selected plant was found only once, in the  $BC_2$  generation, to be smaller than the average of the five largest values. This corresponded to the only time when the selected plant was not the one with the maximum percentage of homozygous recurrent-parent-genotype. The plant had been selected because it displayed a favorable recombination on one side of the *Bt* locus (Figure 1).

The percentage of homozygous recurrent-parent-genotype of the selected  $BC_1$  plant was almost equal to that of an unselected  $BC_2$ , that of the selected  $BC_2$  was larger than that of an unselected  $BC_3$ , that of the selected  $BC_3$  was barely smaller than that of an unselected  $BC_6$ , and that of the selected  $BC_4$  was equal to that of the "perfect" backcross-derived plant, given the set of markers that was used. Such rates of recurrent parent genotype recovery are consistent with results of simulation analyses. Jarboe *et al.* (1994) who used the maize genome as a model reported that three backcross generations and 80 markers were needed to recover 99% of recurrent parent genotype.

#### Number of donor chromosome segments

The number of heterozygous chromosomal segments decreased from one backcross generation to the next. Plants selected at each generation were not necessarily those which had the lowest number of heterozygous chromosomal segments (Table 1). However, with the set of markers used,  $BC_3$  and  $BC_4$  plants were recovered which contained only one heterozygous chromosomal segment: that comprising the *Bt* locus.

#### Linkage drag

Linkage drag around the *Bt* locus was estimated, relative to the length of chromosome 1. Its value was found to lie between 24.0 and 48.4% for the selected  $BC_1$  individual, between 17.6 and 34.8% for the selected  $BC_2$ , between 2.0 and 24.0% for the selected  $BC_3$ , and between 0.0 and 8.4% (respectively 0.0 and 14.5 cM) for the selected  $BC_4$ .

The two values given for each  $gc$  correspond to extreme positions of flanking the transgene construct locus.  $BC_4$  is likely to be less than 1.3% appear to be somewhat high, reflects drag, it is much lower than what (Stam and Zeven 1981; Tanksley *et al.* of tomato cultivars obtained by a 1: Tanksley (1989) found that the sizes cM.

#### Conclusion

These results clearly demonstrate quality advantages over classical through backcrossing. Only four years than a year and a half from plant genotypically fully converted. New genotype could proceed even faster appropriate protocol and resources allocated.

Comparison of  $BC_4$ -derived 1 markers and agronomic performance order to confirm the completeness of

#### References

- ALLARD, R.W. (1960) Principles of plant
- HALLAUER, A.R., and J.B. MIRANDA, University Press, Ames, IA.
- HOSPITAL, F., C.CHEVALET, and P. programs. *Genetics* 132:1199-1210.
- JARBOE, S.G., W.D. BEAVIS, and S.J. O assisted backcross programs by computer on the plant genome. *Scheraga Internat*
- KOZIEL, M.G., G.L. BELAND, C. BO, DAWSON, N. DESAI, M. HILL, McPHERSON, M.R. MECHT, E. MI EVOLA (1993) Field performance of derived from *Lacillus thuringiensis*. *Bt*
- MURRAY, M.G., Y.M.A. J. ROMERO: fragment length polymorphism what

homozygous recurrent-parent-genotype. The relative length of the chromosome between the two flanking markers chosen.

parent-genotype of the BC<sub>1</sub> generation was explained by linkage drag around the *Bt* locus based only on plants selected for two generations the mean percentage of recurrent parent genotype was higher than what would have been expected (Figure 2).

parent-genotype of the selected plant (Table 1) were always very similar to the expected value (Figure 2). The percentage of recurrent parent genotype of the selected plant was found only once, in the five largest values. This corresponded to one with the maximum percentage of recurrent parent genotype that had been selected because it displayed a value (Figure 1).

parent-genotype of the selected BC<sub>1</sub> plant of the selected BC<sub>2</sub> was larger than that of an unselected plant. The percentage of recurrent parent genotype of the "perfect" backcross-derived plant was smaller than that of an unselected plant. The rates of recurrent parent genotype were lower than those of the unselected plants. Jarboe *et al.* (1994) who used backcross generations and 80 markers found that the percentage of recurrent parent genotype was lower.

percentages decreased from one backcross generation to the next. The percentages were not necessarily those which were expected for the segments (Table 1). However, with the selected plants recovered which contained only one *Bt* locus.

relative to the length of chromosome was 4% for the selected BC<sub>1</sub> individual, between 2.0 and 24.0% for the selected BC<sub>2</sub> (14.5 cM) for the selected BC<sub>4</sub>.

The two values given for each generation are extreme values of linkage drag, which correspond to extreme positions of the crossing-overs in the marker-defined intervals flanking the transgene construct locus. Therefore the true linkage drag value of the selected BC<sub>4</sub> is likely to be less than 1.3% of the genome. Although this maximum value may appear to be somewhat high, reflecting the limited selection pressure put here on linkage drag, it is much lower than what would be expected from classical backcross programs (Stam and Zeevalk 1981; Tanksley *et al.* 1989). Practically, in a study of *Tm-2* conversions of tomato cultivars obtained by a large number of classical backcross cycles, Young and Tanksley (1989) found that the sizes of the introgressed fragments ranged between 4 and 51 cM.

### Conclusion

These results clearly demonstrate that molecular markers provide important time and quality advantages over classical procedures for the production of near-isogenic lines through backcrossing. Only four backcross generations were necessary to recover, in less than a year and a half from planting of the BC<sub>1</sub>'s, individuals which appeared to be genotypically fully converted. Nevertheless, it is likely that recovery of recurrent parent genotype could proceed even faster than in the experiment described herein, should the appropriate protocol and resources (population size, number and position of markers) be allocated.

Comparison of BC<sub>4</sub>-derived lines with the recurrent parent for both morphological markers and agronomic performance (including hybrid performance) will be performed in order to confirm the completeness of the conversion.

### References

- ALLARD, R.W. (1960) Principles of plant breeding. Wiley, New York, NY.
- HALLAUER, A.R., and J.B. MIRANDA, JR. (1981) Quantitative genetics in maize breeding. Iowa State University Press, Ames, IA.
- HOSPITAL, F., C. CHEVALET, and P. MULSANT (1992) Using markers in gene introgression breeding programs. *Genetics* 132:1199-1210.
- JARBOE, S.G., W.D. BEAVIS, and S.J. OPENSHAW (1994) Prediction of responses to selection in marker-assisted backcross programs by computer simulation. In: Abstracts of the second international conference on the plant genome. Schering International Inc. 38.
- KOZIEL, M.G., G.L. BELAND, C. BOWMAN, N.B. CAROZZI, R. CRENSHAW, L. CROSSLAND, J. DAWSON, N. DESAI, M. HILL, S. KADWELL, K. LAUNIS, K. LEWIS, D. MADDOX, K. McPHERSON, M.R. MEGHAI, E. MERLIN, R. RHODES, G.W. WARREN, M. WRIGHT, and S.V. SVOLA (1993) Field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis*. *BioTechnology* 11:194-200.
- MURRAY, M.G., Y. MA, J. ROMERO-SEVERSON, D.P. WEST, and J.H. CRAMER (1988) Restriction fragment length polymorphism: what are they and how can breeders use them? In: D. Williams ed.,

58

Proceedings of the 43rd annual corn and sorghum industry research conference. American Seed Trade Association 43:72-87.

STAM, P., and C. ZEVEN (1981) The theoretical proportion of the donor genome in near-isogenic lines of self-fertilizers bred by backcrossing. *Euphytica* 30:227-238.

TANKSLEY, S.D., N.D. YOUNG, A.H.PATERSON, and M.W.SONNERBALE (1989) RFLP mapping in plant breeding: new tools for an old science. *Bio/Technology* 7:257-264.

YOUNG, N.D., and S.D.TANKSLEY (1989) RFLP analysis of the size of chromosomal segments retained around the *Tm-2* locus of inbreds during backcross breeding. *Theor. Appl. Genet.* 77:353-359.

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